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Original Research Article

Methylmercury Biosorption Activity by Methylmercury-resistant Lactic Acid Bacteria Isolated From West Sekotong, Indonesia

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ABSTRACT

Methylmercury has been generally known as a toxic heavy metal for both human and environment. Bacterial-based bioremediation of heavy metal is suggested as an ecofriendly and low-cost bioremediation process. There was limited information regarding the role of lactic acid bacteria (LAB) as detoxification agent for methylmercury addressed for human body. West Sekotong, West Lombok, Indonesia, is one of the newly developed artisanal and small-scale gold mining site with high mercury contamination level. This present study was aimed to isolate the human origin methylmercury-resistant LAB and further evaluate their ability to absorb methylmercury. Methylmercury absorption assay was conducted in broth media. The remaining and absorbed methylmercury was measured using the gas chromatography flame ionization detector. A total of 56 methylmercury-resistant LAB isolates were isolated from 37 feces and 19 breast milk samples from 19 volunteers in West Sekotong. Of them, 10 isolates were further selected based on several basic probiotic characteristics and subjected to methylmercury removal assay. The selected isolates showed different methylmercury absorption ability ranged between 17.375 and 51.597 μg/g of wet mass of cell after incubated for 24 hours. Two isolates from feces showing the best removal activity were identified as *Enterococcus durans* and one isolates from breast milk as *Enterococcus faecium* based on the sequences of 16s rDNA.

1. Introduction

Recent data from the United Nations Environment Programme mentioned that the source of environmental mercury contamination is dominated by artisanal and small-scale gold mining (ASGM) (UNEP, 2000). The number of ASGM rose rapidly during 2006–2009 in Indonesia. District of Sekotong, Located in West Lombok, is one of the newly developed ASGM site in Indonesia that was first started in the middle of 2009 (Ismawati, 2010). Some recent studies had reported the negative output of the ASGM activity in Sekotong on environment and the miner's health (Krisnayanti et al., 2012; Ekawanti & Krisnayanti, 2015).

During the gold extraction, inorganic mercury is commonly used for amalgamation process. Because there is no appropriate waste management in ASGM, the excess mercury directly flows to the soil, ground water, and even sea. Mediated by microbial methylation reaction, that inorganic mercury is transformed into organic mercury, the most toxic form of mercury, such as methylmercury, phenyl mercury, and ethyl mercury and accumulated in living organism including human body (Zhang et al., 2012; Friberg & Mottet, 1989).

Bioremediation using bacterial cell had been introduced for many years and suggested as an ecofriendly and low-cost bioremediation agents (Alluri et al., 2007; Halttunen et al., 2007). However, most published bacterial bioremediation studies were designed for waste management. Lactic acid bacteria (LAB), the major bacteria group used for probiotic, exhibits many beneficial effects for human health. Some strains of LAB were reported to have ability to remove cadmium, arsenic, lead, mercury, and further decrease their toxicity (Halttunen et al., 2007; Bhakta et al., 2010; Abdel-Salam et al., 2012; Zhai et al., 2015; Allam et al., 2015). There was limited information regarding the role of LAB in methylmercury detoxification addressed for human body. To meet the

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future goal for the alternative safety methylmercury detoxification agent for human consumption, we isolated the human origin methylmercury-resistant LAB from breast milk and feces, then further investigated the methylmercury biosorption activity.

2. Materials and Methods

2.1. Sample sources

Ten breast milk and 12 stool samples obtained from 19 subjects from Gawah Pudak Village and Tembowong Village (Figure 1) were used as bacterial sources in this study. All the subjects were apparently healthy adults aged between 18 and 35 years, have been living in Sekotong area for more than 5 years, and did not consume any antibiotic for at least 2 months before sample collection. The procedure of sample collection had been accepted by Medical and Health Research Ethics Committee of Faculty of Medicine, Universitas Gadjah Mada.

2.2. Isolation of methylmercury-resistant bacteria

Isolation of methylmercury-resistant bacteria was divided into two steps: first, isolation was performed using De Man, Rogosa, and Sharpe agar (MRSA) supplemented with 5 μg/mL of mercury chloride and followed by repurification onto MRSA supplemented with 5 μg/mL of methylmercury chloride. Each breast milk (1 mL) and stool samples (1 g) were diluted into 0.85% of sodium chloride to reach the appropriate dilution factor (10^4–10^8), inoculated into the agar plate, and then incubated anaerobically for 24–48 hours at 37°C. The morphologically different colonies were purified into MRSA containing 5 μg/mL of methylmercury, then streaked the growing colony into the fresh MRSA.

2.3. Screening of probiotic potential LAB

Screening of probiotic potential LAB was performed according to several basic criteria of probiotics. Gram staining and catalase test were conducted to screen the LAB. Afterward, all the gram-positive isolates and those showing negative result on catalase test were subjected to further screenings, which were resistance assay in low pH medium, resistance assay in bile salt supplemented media, and antimicrobial activity assay against some pathogenic bacteria. Isolates showing appropriate growth after 6 hours of incubation in low pH and bile salt media and also appropriate antimicrobial activity were selected for further investigation.

2.4. Methylmercury biosorption assay

Methylmercury biosorption activity of the selected isolates was evaluated in De Man, Rogosa, and Sharpe Broth containing 10 μg of methylmercury chloride. Approximately, 10^6 CFU/mL of each isolate culture was inoculated into the tested medium and incubated for 24 hours at 37°C. Thereafter, the culture was centrifuged for 15 minutes at 3,500*g*. The pellet and supernatant were separated and then subjected for methylmercury measurement using the gas chromatography flame ionization detector.

2.5. Identification of selected isolates

Three isolates showing the best methylmercury biosorption activity were further identified according to the sequences of 16s rDNA. The genomic DNA was extracted using microbial DNA isolation kit. Before DNA extraction, the isolate was cultured into De Man, Rogosa, and Sharpe Broth for 20–24 hours at 37°C. DNA amplification was performed using forward primer 5′-AGAGTTTGATCMTGGCTCAG-3′ and reverse primer 5′-GGTTACCTTGTTACGACTT-3′. The amplification condition was as follows: 96°C for 4 minutes as initial denaturation, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 52°C for 1.5 minutes, extension at 68°C for 8 minutes, and a final extension at 68°C for 10 minutes. The amplicons then were sequenced by 1st Base (Singapore). The sequencing results were aligned with some similar sequences obtained from GenBank database. The neighbor-joining analysis was used to construct the phylogeny tree.

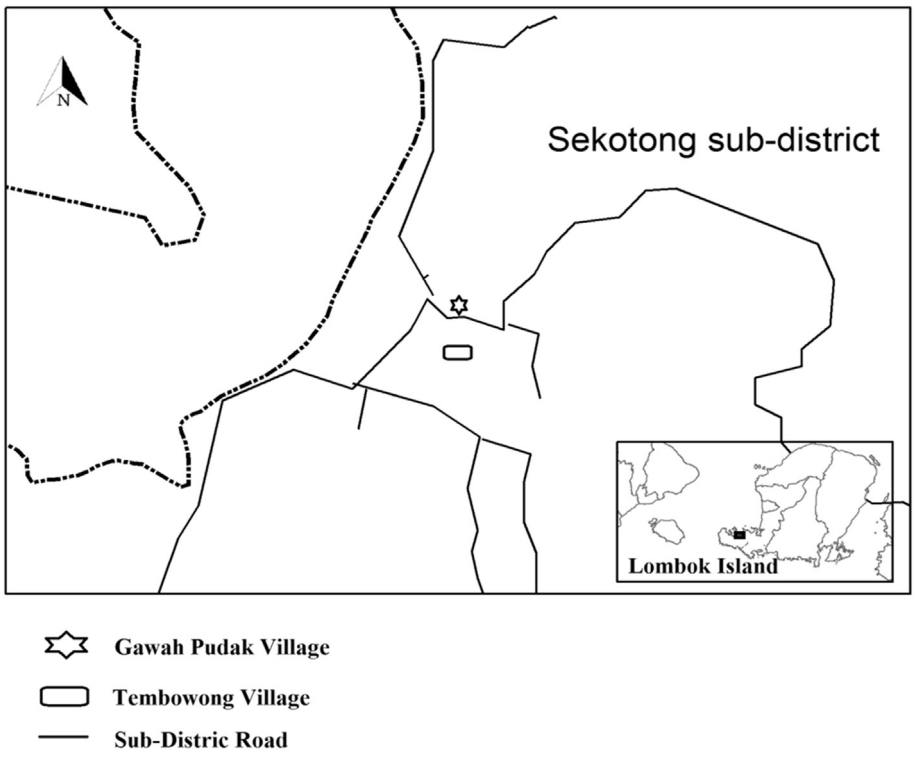

Figure 1. Sampling site location: Gawah Pudak and Tembowong Village.

3. Results

3.1. Isolation and screening of probiotic potential LAB

A total of 71 isolates of methylmercury-resistant bacteria were successfully isolated. Among them, 45 isolates were isolated from feces, whereas 26 isolates from breast milk (Table 1). The breast milk origin isolates in this study had lower resistance toward low pH and bile salt (data not shown). Therefore, at the end of the screening, only one isolate was selected as the representative of breast milk origin isolate (Table 1).

3.2. Methylmercury biosorption assay

Methylmercury biosorption assay was conducted in broth media containing 10 μg of methylmercury chloride. Table 2 represents the methylmercury biosorption activity of the selected isolates. Methylmercury resistance is closely related to detoxification/remediation ability. All the selected methylmercury-resistant isolates showed methylmercury biosorption activity with biosorption capacity ranged between 17.375 and 51.597 μg/g of cell pellet. The highest biosorption activity was shown by FG11 75B followed by AG03 52A and FG11 85F. Within 24 hours of incubation, the methylmercury in media was reduced up to 70%, but only about 23.79%–49.75% were absorbed in cell pellet.

3.3. Identification of selected isolates

Three isolates showing the best methylmercury biosorption capacity: FG11 85F, FG11 75B and AG03 52A were selected for species identification. Based on the 16s rDNA sequence, FG11 85F and FG11 75B were identified as *Enterococcus durans*, whereas AG03 52A was identified as *Enterococcus faecium*. The similarity of these isolates with the reference strain was $\geq$ 99%. Figure 2 shows the phylogeny tree of the three selected isolates with some similar strains.

4. Discussion

The human origin mercury–resistant bacteria had been reported before. However, most of them were oral and gut microflora (Edlund et al., 1996; Leistevuo et al., 2000; Stapleton et al., 2004; Nygren et al., 2014). Based on our knowledge, there was no previous study reported the methylmercury-resistant bacteria isolated from breast milk. Reported by several studies, inorganic and organic mercury such as methylmercury are also accumulated in breast milk, thus methylmercury-resistant bacteria could be also isolated from breast milk, as isolated in this study (Bjornberg et al., 2005; Clarkson et al., 2007; Bose-O'Reillya et al., 2008). Mercury accumulated in some parts of human body such as hairs, nails, and also breast milk were suggested as the indicator of mercury uptake orally through daily consumption (Clarkson et al., 2007). Because mercury is the important chemical used in gold amalgamation process, the level of mercury contamination is commonly found linear with the development of ASGM and has become a global concern. The relationship between mercury–resistant bacteria and the level of mercury accumulated in human body had been also reported (Leistevuo et al., 2000). Therefore, the presence of methylmercury-resistant bacteria with high methylmercury biosorption activity found in this study might indicate the high level of methylmercury accumulated in the body of the subjects.

Table 2. Methylmercury removal activity

Isolates	Sources	Methylmercury remained in supernatant (μg)*	Methylmercury adsorbed in cell (μg)	Methylmercury adsorption capacity (μg/g cell)
FG03 75A	Feces	3.288	3.980	43.312
FG05 72A	Feces	4.845	3.461	27.542
FG08 72B	Feces	4.023	3.374	21.086
FG08 75A	Feces	3.331	2.379	17.375
FG11 75B	Feces	4.802	4.888	51.597
FG11 85A	Feces	2.942	4.066	33.556
FG11 85E	Feces	3.807	4.975	42.651
FG11 85F	Feces	5.580	3.374	46.240
FG11 85G	Feces	2.898	4.456	45.829
AG03 52A	Breast milk	4.196	4.412	47.704

* Initial methylmercury concentration in media was 10 μg. Values are the mean of two replicates..

Heavy metal–resistant bacteria have been studied extensively to be implemented as bioremediation agents. For human body, the common therapy of heavy metal poisoning is chemical chelating agent like ethylenediaminetetraacetic acid (EDTA) (Flora & Pachauri, 2010). LAB showing high resistance to heavy metal has been suggested as the alternative biology chelating and detoxification agent (Bhakta et al., 2012; Gourdon et al., 1990; Kinoshita et al., 2013; Zoghi et al., 2014). Had ability to survive in high methylmercury concentration and to absorb or/and accumulate the methylmercury in their cells, the methylmercury-resistant LAB isolates found in this study are suitable to be used for remediation purpose.

Absorption of heavy metal by living organism such as bacteria known as biosorption had been reported by several previous studies (Karunasagar et al., 2003; Zouboulis et al., 2004; Lua et al., 2006; Congeevaram et al., 2007; Bhakta et al., 2012). Negative charge of bacterial cell wall is predicted able to bind the cationic heavy metal. Moreover, it was suggested that gram-positive bacteria like LAB have higher absorption activity than gram-negative bacteria because of the differences of the cell wall structure (Gourdon et al., 1990). The metal biosorption commonly occurs passively involving the specific binding proteins, yet the dead cells could also absorb the heavy metals (Kinoshita et al., 2013; Zoghi et al., 2014). Unfortunately, in some strain of bacteria, the biosorption activity was reported reversible, followed by desorption reaction. That desorption phenomenon could occur spontaneously, strain dependent, and affected by several external factors (Harvey & Leckie, 1985; Manmaril et al., 1997; Fowle & Fein, 2000).

Despite physical biosorption, bacterial enzymatic activity also plays an important role in methylmercury bioremediation process. Bacterial mercury resistance and detoxification ability are supported by several enzymes encoded by genes harbor in mer operon

Table 1. Methylmercury-resistant bacteria obtained from feces and breast milk

Sources of isolates	Number of isolates					
	First isolation in mercury chloride media	Purification in methylmercury chloride media	Screening for LAB	Selection of basic criteria of probiotic		
				Low pH tolerance	Bile salt tolerance	Antimicrobial activity
Feces	45	45	37	24	15	9
Breast milk	26	26	19	7	2	1
Total	71	71	56	31	17	10

LAB = lactic acid bacteria.

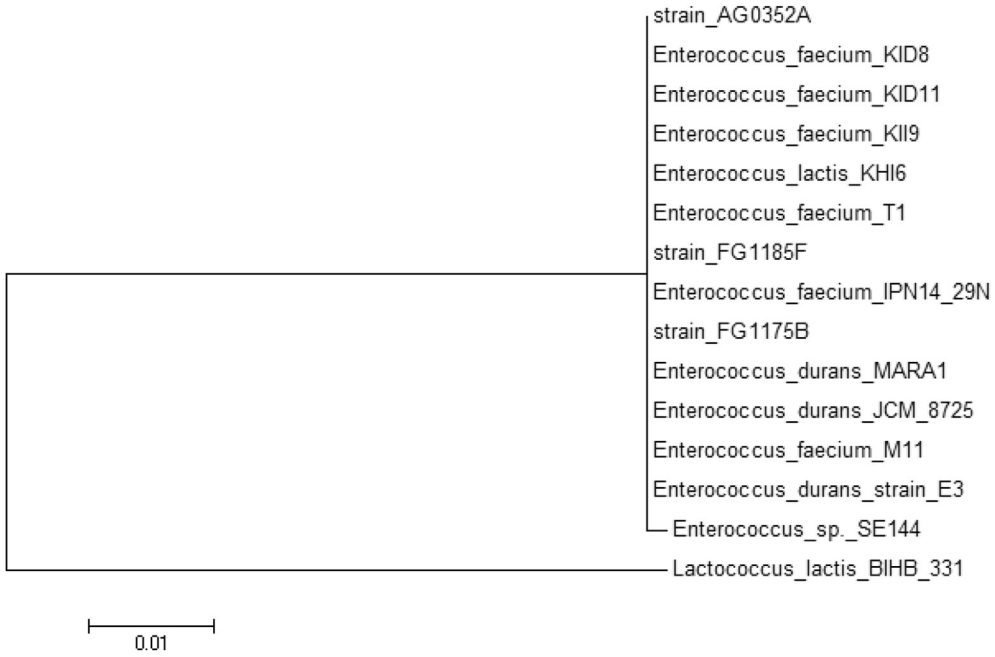

Figure 2. Phylogeny tree of the three selected isolates constructed using neighbor-joining analysis based on the 16s rDNA sequences.

(Moore et al., 1990; Chien et al., 2010; Osborn et al., 1997; Mathema et al., 2011). Organomercurial lyase encoded by merB gene is the major suggested enzyme contributed in methylmercury resistance and detoxification. The enzyme catalyzes the demethylation of methylmercury and commonly followed by reduction reaction by mercuric reductase enzyme (Chien et al., 2010; Reniero et al., 1995; Schaefer et al., 2004). In this study, we found that the sum of mercury accumulated in the cell pellet and remained in supernatant did not reach 10 μg, the initial methylmercury concentration. These results suggest that the biosorption was not the only one mechanism contributed in methylmercury removal. Enzymatic reactions and other mechanism were also probably took part in the methylmercury removal activity. Both biosorption and enzymatic heavy metals removal process were strain dependent and affected by several external factors such as pH and contact time (Halttunen et al., 2007; Gourdon et al., 1990).

With those explained possible mechanisms, this study highlighted the important role of gut microbiota in human xenobiotic metabolism, especially for methylmercury. The selected isolates obtained in this present study will be subjected for several further studies to achieve the future goal for the development of the methylmercury detoxification agent. Further safety assessments are also required to be accepted for human consumption including the investigation of the antibiotic resistance and the toxic capacity.

In conclusion, this present study shows the potency of the human origin methylmercury-resistant LAB isolates to remove the methylmercury accumulated in aqueous system. The removal activity was proposed as cumulative function of passive cellular absorption and other mechanism, probably enzymatic reaction. Limited data show the methylmercury resistance profile and the detoxification activity of human origin bacteria, thus our study will provide preliminary data of LAB isolated from breast milk and feces for potential methylmercury removal agent.

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